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TITLE: F-box genes and proteins

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Drawing Description Text (4):
FIG. 2A shows an SDS-PAGE analysis of purified Cln1 HA/Gst-Cdc28HA/Cks1.

Detailed Description Text (202):
Sic1/Clb5/Gst-Cdc28HA(K-) complexes were purified from 4.times.10.sup.8 cells, as described by Connell-Crowley et al. (Connell-Crowley et al., Mol. Biol. Cell., 8:287-301[1997]). Briefly, eight T-150 flasks of insect cells (Highfive, Invitrogen) were infected with 1 ml each of baculoviruses expressing either GST-Cdc28HA, Cln1HA, Cks1, and Cak1, or baculoviruses expressing Gst-Cdc28HA(K-), Clb5, and Sic1. After 40 hours, the cells were lysed at 4.degree. C., in 6 ml of NETN (20 mM Tris-HCl, pH 8, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 5 mM NaF, 30 mM p-nitrophenylphosphate, 1 .mu.g/ml each leupeptin and antipain, and 1 mM PMSF). Lysates were cleared by centrifugation at 14,000.times.g for 10 minutes. Supernatants were rotated with 0.2 ml of GSH-Sepharose for 60 minutes at 4.degree. C., and the beads were washed three times with 2 ml of the lysis buffer, followed by two washes with 100 mM Tris (pH 8), 100 mM NaCl. Proteins were then eluted with 0.2 ml of 100 mM Tris (pH 8), 100 mM NaCl, 40 mM glutathione (Sigma), and 10% glycerol. The proteins were then stored at -80.degree. C. until use.

Detailed Description Text (203):
B. Gst-Cdc28HA/ClnHA/Cks1 and Gst-Cdc28HA(K-)/ClnHA/Cks1 Complexes

Detailed Description Text (204):
Gst-Cdc28HA/ClnHA/Cks1 (i.e., "ClnHA/Gst-Cdc28HA/Cks1" in the legend for FIG. 2A) and kinase impaired Gst-Cdc28HA(K-)/Cln1HA/Cks1 complexes were prepared as described above, as were cells co-infected with viruses expressing appropriate proteins, and CAK1 expressing virus generated from a cDNA generously provided by C. Mann (See, Thuret et al., Cell 86:565-576 [1996]). The presence of Cks1 and Cak1 resulted in a 5-fold increase in the yield of active Cln/Cdc28 kinase complexes, as purified after insect cell co-infection (determined using histone HI as a substrate). FIG. 2A shows an SDS-PAGE analysis of purified Cdc28HA/Cks1. In this Figure, the asterisk indicates the position of endogenous GST protein.

Detailed Description Text (206):
Phosphorylated Sic1 complexes were generated by incubating 2.5 .mu.M Sic1/Clb5/Gst-Cdc28HA(K-) with Gst-Cdc28HA/Cln1HA/Cks1 (50 nM) and 1 mM ATP in kinase buffer (50 mM Tris HCl (pH 7.5), 50 mM NaCl, 10 mM MgCl.sub.2) for 45 minutes at 25.degree. C. Control unphosphorylated Sic1 complexes were produced in an identical fashion by omitting Cln1 kinase. Cln/Cdc28 autophosphorylation was performed by incubating 200 nM Cln/Cdc28 complexes with 1 mM ATP in kinase buffer at 25.degree. C. for 1 hour. To generate phosphorylated Sic1 free of Cln/Cdc28 kinase, bacterial Sic1 (0.5 .mu.M) was incubated with 2 mM ATP and Cln2/Gst-Cdc28/Cks1 immobilized on GST-Sepharose (Pharmacia) for 60 minutes at 37.degree. C. Forty ng of phosphorylated Sic1 were removed from the beads for use in ubiquitination reactions, at a final concentration of 1 nM. For .sup.32 P-labeling of Sic1 and Cln1 proteins, kinase reactions were performed at 25.degree. C. for 30 minutes, using 50 .mu.M

(.gamma.-.sup.32 P ATP (0.3 nCi/pmol) followed by incubation with 1 mM unlabeled ATP for an additional 30 minutes.

Detailed Description Text (256):
In these experiments, 0-100 .mu.g YFII (a 250 mM NaCl eluate from a DEAE-cellulose column prepared exactly as described in Deshaies et al. [1995], supra) was supplemented with 500 nM Cdc34, 100 nM human E1, ubiquitin, and an ATP regenerating system (2 mM ATP, 600 mM creatine phosphate, and 0.15 mg/ml creatine kinase). The ubiquitination reaction was initiated by addition of 20 ng Cln1HA/Gst-Cdc28HA/Cks1. After incubation for 60 minutes at 25.degree. C., the reactions were quenched and immunoblotted with anti-HA antibodies to detect Cln1HA and Gst-Cdc28HA. In FIG. 5E, the protein indicated by an asterisk is a yeast protein in YFII that cross-reacts with the anti-HA antibodies used. As indicated in this Figure, this preparation of Cln1 is competent for ubiquitination.

Detailed Description Paragraph Table (1):
TABLE 1 Baculovirus Expression Vectors Virus Tag Base Vector Cak1 None pVL Cdc4 None pBBIII Cdc4.DELTA.WD None pBBIII Cdc4.sup.F C-terminal Flag pBBIII Cdc34 None pBBIII Cdc53.sup.M N-terminal Myc pBBIII Clb5 None pVL Cln1.sup.HA C-terminal HA pBBIII Cln2.sup.HA C-terminal HA pVL Gst-Cdc28.sup.HA N-terminal Gst pVL C-terminal HA Gst-Cdc28.sup.HA (D154N) N-terminal Gst pVL C-terminal HA Grr1.sup.G10 N-terminal His.sub.6 -G10 pBBHis His.sup.6 -Cks1 N-terminal His6 pVL Sic1 None pBBIII Skp1 None pVL Skp1.sup.F N-terminal Flag pBBIII Gst-Skp1 N-terminal Gst pVL